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METHOD FOR ENZYMATIC SPLITTING OF RUTINOSIDES

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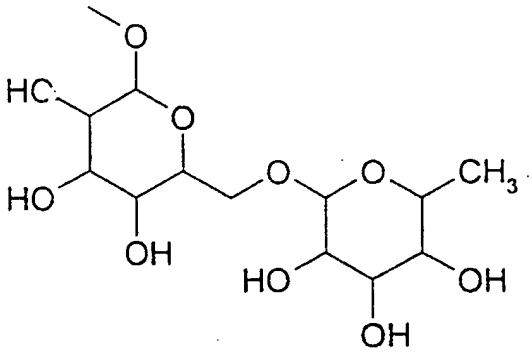
(57) Abstract

Disclosed is a method for enzymatic splitting of rutinosides, whereby rhamnose and/or corresponding glucopyranosides is/are obtained . The inventive method is carried out in the presence of a solvent mixture made up of water and one or several organic solvents.

The invention concerns a method for enzymatic cleavage of rutinosides while obtaining rhamnose and/or the corresponding glucopyranosides, where the reaction is carried out in the presence of a solvent mixture of water and one or more organic solvents.

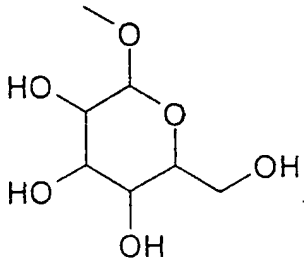
Within the scope of this invention compounds that contain a sugar-free component to which a residue of formula (I)



(I)

is bound via a glycosidic bond are called rutinosides. For example, rutinosides are flavonoids with the bisglycosidic unit shown in formula I. Rhamnose and/or the corresponding glucopyranosides are obtained from the rutinosides by the method in accordance with the invention.

The glucopyranosides are derived from the rutinosides in that they contain a residue of a formula (I*)



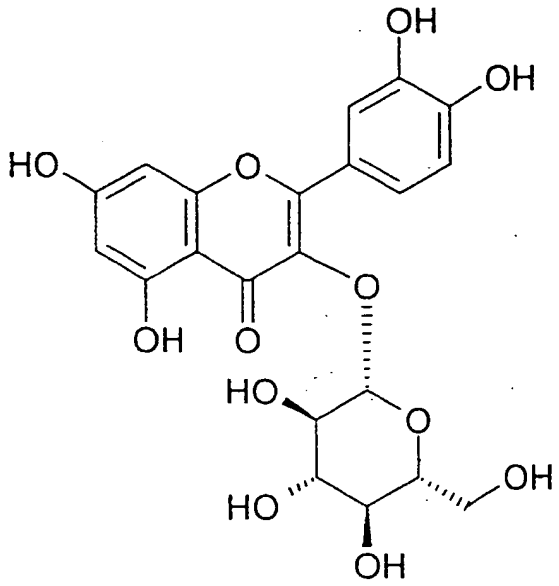
(I*)

bonded to the sugar-free component instead of the residue of formula (I). For example, according to the method in accordance with the invention both rhamnose as well as isoquercetin can be obtained from rutin.

Rhamnose is a monosaccharide that is very common in nature, but that for the most part only occurs in small amounts. An important source of rhamnose consists, for example, of the

glycosidic residues of natural flavonoids like rutin, from which rhamnose can be obtained by glycoside cleavage. Rhamnose plays an important role as a starting material for the preparation of synthetic flavorings like furaneol, for example.

Isoquercetin is a monoglycosidated flavonoid with the following structural formula (II).

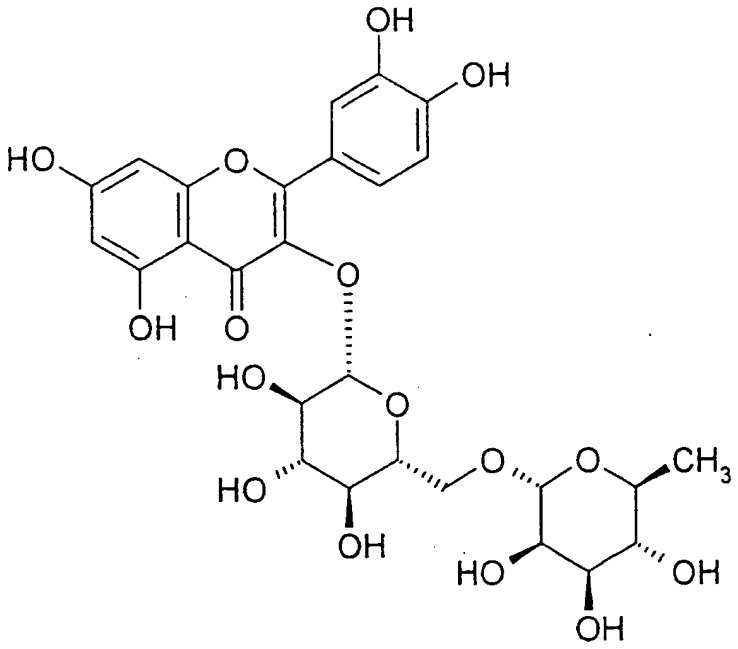


(II)

Flavonoids (lat. flavus = yellow), which are very common colorants in plants, are, for example, glycosides of flavones, to which the backbone of flavone (2-phenyl-4H-1-benzopyran-4-one) is common. The sugar-free component of the flavonoids is the so-called aglycone. For example, isoquercetin is a glycoside of the aglycone quercetin (2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-1-benzopyran-4-one), which differs from flavone by the presence of five hydroxyl groups. In isoquercetin the carbohydrate residue glucose is bonded to the hydroxyl group in position 3 of the quercetin. Isoquercetin is called quercetin-3-O-β-D-glucopyranoside or 2-(3,4-dihydroxyphenyl)-3-(β-D-glucopyranosyloxy)-5,7-dihydroxy-4H-1-benzopyran-4-one, for example. However, it is also called hirsutrin, for example.

Flavonoids or flavonoid mixtures are used, for example, in the food and cosmetics industries and are gaining importance there. Monoglycosidated flavonoids such as isoquercetin are characterized by good ability to be absorbed by the human body.

An example of a naturally occurring flavonoid with a bisglycosidic unit is rutin, which has the following structural formula (III):



(III)

Like isoquercetin, rutin is a glycoside of the aglycone quercetin, where the carbohydrate residue rutinoside is bonded to the hydroxyl group in position 3 of the quercetin. The carbohydrate residue in rutin consists of a glucose and a terminally bonded rhamnose, or 6-deoxymannose unit, linked in positions 1 and 6. Rutin is called, for example, quercetin-3-O-β-D-rutinoside or 2-(3,4-dihydroxyphenyl)-3-{[6-O-(6-deoxy-α-mannopyranosyl)-β-D-glucopyranosyl]oxy}-5,7-dihydroxy-4H-1-benzopyran-4-one. However, it is also known under the names sophorin, birutan, rutabion, taurutin, phytomelin, melin or rutinoside, for example.

Rutin forms pale yellow to greenish needles with three molecules of water of crystallization. Water-free rutin has the property of a weak acid, turns brown at 125°C and decomposes at 214-215°C. Rutin, which occurs in many plant species, frequently together with vitamin C, for example, in citrus species, in yellow pansies, forsythia and acacia species, various solanum and nicotiana species, capers, linden flowers, St. John's wort (tea etc.), was isolated in 1842 from common rue (Ruta graveolens). Rutin can also be obtained from the leaves of buckwheat and the East Asian drug Wei-fa (Sophora japanoca, Fabaceae), which contains 13-27% rutin.

For the reasons given above, it is desirable to produce both rhamnose as well as monoglycosidated flavonoids from natural raw materials, for example, from flavonoids that have a bisglycosidic unit. In this connection, the cleavage of rutinosides to rhamnose and the corresponding glucopyranosides, for example, is of interest.

Enzymatically catalyzed preparations of rhamnose have been described in the literature. For example, EP 0 317 033 describes a method for producing L-rhamnose, where the rhamnosidic bonding of glycosides that contain rhamnose in terminal position is achieved through enzymatic hydrolysis. To be sure, such cleavages of glycosides with bisglycosidic carbohydrate residue that are carried out in aqueous media are for the most part not very selective. For example, for the most part a mixture of the monosaccharides glucose and rhamnose is formed because of the bisglycosidic structure of the carbohydrate residue in the rutin. Moreover, for the most part high fractions of the aglycone quercetin as well as other undesirable byproducts are formed.

In addition, enzymatically catalyzed cleavages of rutin have also been described in JP 01213293, for example. However, such reactions that are carried out in aqueous media are for the most part also not highly selective.

Therefore, there was the task of developing a method for enzymatic cleavage of rutinosides to obtain rhamnose and/or the corresponding glucopyranosides, which avoids or at least reduces the disadvantages of the known methods and in particular enables preparation of rhamnose and the glucopyranosides that is as selective as possible, so that these products can be produced with high yield.

Surprisingly, it was now found that this task is solved if the method for enzymatic cleavage of rutinosides while obtained rhamnose and/or the corresponding glucopyranosides is carried out so that the reaction takes place in a solvent mixture of water and one or more organic solvents.

The method in accordance with the invention is especially characterized by the fact that the cleavage of the rutinosides to rhamnose and the corresponding glucopyranosides takes place with high selectivity. According to the method in accordance with the invention, preferably rhamnose and the glucopyranosides are obtained by suitable further processing. In addition, by the method in accordance with the invention either only rhamnose or only the glucopyranosides can be obtained by suitable further processing. This invention makes available an advantageous method for enzymatic cleavage of rutinosides while obtaining rhamnose and/or the corresponding glucopyranosides. According to this method the rutinoside is brought into contact with a catalytic quantity of an enzyme in a solvent mixture of water and one or more organic solvents. Preferably, the reaction is carried out with good mixing, for example, by stirring.

The reaction is preferably carried out under a nitrogen atmosphere.

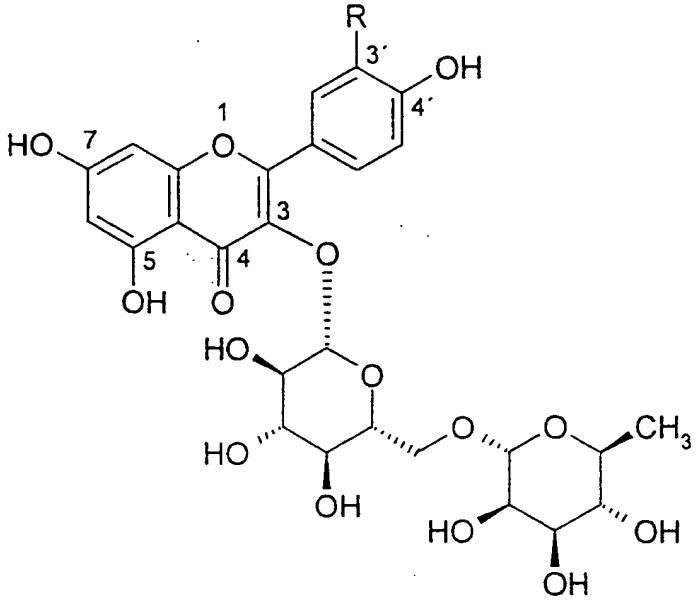
Suitable rutinosides for the method in accordance with the invention are, for example, rutinosides that contain as the sugar-free component or aglycone a 2-phenyl-4H-1-benzopyran-4-one parent substance that has a residue of formula (I) in position 3 and the phenyl groups of

which, apart from position 3, can also be substituted one or more times by OH or O-$(CH_2)_n$-H, where n means 1-8.

Preferably, n means 1.

The substitution of the 2-phenyl-4H-1-benzopyran-4-one parent substance by OH or O-$(CH_2)_n$-H preferably occurs in position 5, 7, 3' and/or 4'.

Especially preferred rutinosides corresponds to formula (IV):



(IV)

in which R means H (campherol rutinoside), OH (rutin) or OCH_3 (isorhamnetin rutinoside). According to the method in accordance with the invention rhamnose and campherol glucoside can be obtained from campherol rutinoside, rhamnose and isoquercetin can be obtained from rutin, and rhamnose and isorhamnetin glucoside can be obtained from isorhamnetin rutinoside. The rutinoside rutin is especially preferably used.

The invention also concerns the use of campherol glucoside, isoquercetin and/or isorhamnetin glucoside in the food and cosmetics industries.

The method in accordance with the invention does not require highly pure educts. For example, mixtures of rutinosides can also be used for the method in accordance with the invention. The reaction can be successful, for example, even if the educt is contaminated by other flavonoids. For example, it can also be carried out with mother liquor residues from rutin production.

Suitable enzymes for the method in accordance with the invention are hydrolases. Hydrolases that are obtained from the strain Penicillium decumbens, especially the enzymes naringinase and hesperidinase, are preferably used. The enzyme naringinase is really most highly preferred.

The educts and enzymes for the method in accordance with the invention are commercially available or can be obtained or prepared by methods that are well known to specialists.

Suitable reaction temperatures for the methods in accordance with the invention are temperatures between 15 and 80°C. Preferably, the method in accordance with the invention is carried out at reaction temperatures of 30-50°C, especially at reaction temperatures of 35-45°C.

If the reaction temperature is too low, the reaction will progress an at unreasonably slow reaction rate. On the other hand, if the reaction temperature is too high, the enzyme, which is a protein, will become denatured and thus deactivated.

Suitable pH values for the method in accordance with the invention are pH values between 3 and 8. Preferably, the method in accordance with the invention is carried out at pH values of 4.5-7, especially at pH values of 4.8-6.8. Furthermore, however, preferred pH values can vary within the given limits in each case according to the enzyme that is used. For example, pH values of 6.4-6.8 are really most highly preferred when using the enzyme naringinase.

Preferably, the method is set up so that the pH is adjusted with the help of a buffer system. In principle, all buffer systems that are suitable for adjusting the pH values indicated above can be used. However, an aqueous citrate buffer is preferably used.

Preferably, the preferred temperature and pH ranges are combined, i.e., the reaction is preferably carried out at a reaction of 15-80°C and at a pH value of 3-8, especially preferably at a reaction temperature of 30-50°C and at a pH value of 4.5-7, and especially preferably at a reaction temperature of 35-45°C and at a pH value of 4.8-6.8.

The organic solvent or solvents that are present in addition to water consist of both organic solvents that are miscible with water as well as organic solvents that are immiscible with water.

Suitable organic solvents for the method in accordance with the invention are nitriles like acetonitrile, amides like dimethylformamide, esters such as acetates, especially methyl acetate or ethyl acetate, alcohols such as methanol or ethanol, ethers such as tetrahydrofuran or methyl tert-butyl ether and hydrocarbons such as toluene. Preferably, the method in accordance with the invention is carried out in the presence of one or more organic solvents from among acetates, methanol, ethanol, methyl tert-butyl ether, or toluene. Especially preferably, the method in accordance with the invention is carried out in the presence of one or more acetates, especially in the presence of methyl acetate.

Suitable volume ratios of water to organic solvents for the method in accordance with the invention are ratios from 1:99-99:1. Preferably the method in accordance with the invention is carried out at water:organic solvent volume ratios of 20:80-80:20, especially at volume ratios of 50:50-70:30.

Suitable weight ratios of rutinoside to (water + organic solvent) for the method in accordance with the invention are ratios from 0.001:99.999-40:60. Preferably, the method in accordance with the invention is carried out at rutinoside:(water + organic solvent) weight ratios of 0.005:99.995-20:80, especially at weight ratios of 0.5:99.5-10:90.

Suitable enzyme:rutinoside weight ratios for the method in accordance with the invention are ratios from 0.005:99.995-50:50. Preferably, the method in accordance with the invention is carried out at enzyme:rutinoside weight ratios of 0.5:99.5-30:70, especially at weight ratios of 2:98-20:80.

The progress or the completion of the reaction can be monitored by means of thin layer chromatography (TLC), for example.

After the end of the reaction the reaction mixture primarily consists of water, organic solvents, buffer (sodium citrate), enzyme, small amounts of unreacted rutinoside, rhamnose, glucopyranoside, small amounts of aglycone of the rutinoside and possibly small amounts of glucose. The isolation of the desired reaction products, rhamnose and glucopyranoside, takes place by current methods. "Usual further processing" within the scope of this invention is understood to mean the following:

Preferably, the organic solvent is distilled out at reduced pressure. The glucopyranoside that crystallizes in this case, which can contain, for example, small amounts of the rutinosides and its aglycone, is separated from the remaining reaction mixture, for example by vacuum filtration or filtration at reduced pressure or by centrifuging the precipitated crystals out. Then the solids are washed, preferably with water, and then dried. The purity of the resulting glucopyranoside is usually greater than 94% when pure rutinoside is used. For further purification it can be recrystallized from suitable solvents, for example from water or from solvent mixtures consisting of toluene or methanol or consisting of water and methyl acetate.

Water, buffer, enzyme, small amounts of rutinoside, small amounts of its aglycone and possibly glucose as well as the desired reaction product rhamnose remain in the filtrate.

Isolation of the rhamnose remaining in the filtrate can be achieved by known methods, for example by ultrafiltration, by passing the filtrate over cation and/or anion exchangers, by crystallization, and by mechanical separation such as filtration. Glucose that is possibly present in the filtrate can be separated by yeast fermentation, for example.

The substances that accumulate in the processing steps, for example the organic solvent, enzyme or buffer, for example sodium citrate, can be recycled and thus used for further reactions.

Analysis of the reaction products can be done by HPLC, for example using standard HPLC apparatus and columns containing reversed phase materials with C_{18} alkyl coating.

The following examples are intended to illustrate this invention. However, they are not in any way to be interpreted as limiting.

Examples

The sources for the substances that were used are as follows:

Rutin:	Merck KGaA, Article No. 500017
Naringinase:	Sigma, Article No. N-1385
Hesperidinase:	Amano, Article No. HPV 12519
Citric acid monohydrate:	Merck KGaA, Article No. 100243
Sodium hydroxide:	Merck KGaA, Article No. 105587
Methyl acetate:	Merck KGaA, Article No. 809711

The reaction was monitored by thin layer chromatography (TLC) and the reaction products were analyzed by HPLC.

TLC Conditions:

TLC plates:	Silica gel 60 (Merck KGaA, Article No. 105719),	
Eluent:	mixture of ethyl acetate:ethyl methyl ketone:formic acid:water: 1-butanol in a 50:30:10:10:5 volume ratio,	
Spray reagent:	Iodosulfuric acid,	
Detection:	UV light (254 nm),	
R_f values:	Rutin:	0.38,
	Isoquercetin:	0.61,
	Quercetin:	0.96.

HPLC conditions using a standard HPLC unit:

Cartridge:	LiChroCart® 250/4 with
Column:	LiChroSorb® RP18 (reversed phase material with C18 alkyl coating and particle size 5 µm (Merck KGaA, Article No. 151355)),
Eluent:	mixture of acetonitrile and water in 20:80 volume ratio (pH 2; buffered with NaH2PO4 · H2O/H3PO4),
Flow rate:	1 mL/min
Wavelength:	260 nm,
Temperature:	30°C,

Sample volume:	10 µL	
Sample preparation:	5 mg of the sample dissolved in 3 mL methanol and filled with eluent to 10 mL	
Retention times:	Rutin:	7-7.5 min,
	Isoquercetin:	8.5-9 min,
	Quercetin:	40-43 min.

Example 1

3.15 g citric acid monohydrate is dissolved in 150 mL demineralized water and adjusted to the pH of 6.6 with 10 g 32% aqueous sodium hydroxide. Then 150 mL methyl acetate is added and 5.0 g rutin and 0.5 naringinase are added under nitrogen atmosphere while stirring (200 rpm). Then the reaction mixture is stirred for 24 h at a reaction temperature of 40°C. After conventional further processing rhamnose and 3.82 g yellow crystals are obtained. Analysis of the yellow crystals by HPLC gives the following composition:

Rutin: 1.2 area percent,
Isoquercetin: 94.4 area percent.
Quercetin: 2.6 area percent.

Example 2

0.32 g citric acid monohydrate is dissolved in 150 mL demineralized water and 150 mL methyl acetate is added. Then the emulsion is adjusted to the pH of 5.0 with 2.5 g 1-normal aqueous sodium hydroxide and 5.0 g rutin and 0.125 g hesperindinase are added under a nitrogen atmosphere. Then the reaction mixture is stirred for 21 h at a reaction temperature of 40°C (250 rpm). After conventional further processing rhamnose and 3.41 g yellow crystals are obtained. Analysis of the yellow crystals by HPLC gives the following composition:

Rutin: 0.1 area percent,
Isoquercetin: 98.0 area percent.
Quercetin: 0.2 area percent.

Example 3

6.37 g citric acid monohydrate is dissolved in 300 mL demineralized water and adjusted to the pH of 6.6 with 11.33 g 32% aqueous sodium hydroxide. Then 300 mL methyl acetate is added and 20.11 g of an educt mixture that consists of 53.5 area percent rutin, 39.8 area percent isoquercetin, and 0.4 area percent quercetin (mother liquor residue from rutin production), and 1.11 g naringinase are added under a nitrogen atmosphere. Then the reaction mixture is stirred for 46 h at a reaction temperature of 40°C (200 rpm). After conventional further processing

rhamnose and 14.18 g yellow crystals are obtained. Analysis of the yellow crystals by HPLC gives the following composition:

Rutin:	0.5 area percent,
Isoquercetin:	92.0 area percent.
Quercetin:	4.7 area percent.

Comparative example

12.6 g citric acid monohydrate is dissolved in 600 mL demineralized water and adjusted to the pH of 6.6 with 40 g 32% aqueous sodium hydroxide. Then 10.0 rutin and 1.0 naringinase are added under a nitrogen atmosphere while stirring (200 rpm). After about 24 h of stirring at 36°C isoquercetin and rutin are present in the reaction mixture in a ratio of about 2:1. The mixture is stirred another 7 h at 36°C and 22 h at 40°C and the reaction mixture is then cooled to 15°C. After conventional further processing rhamnose and 7.25 g yellow crystals are obtained. Analysis of the yellow crystals by HPLC gives the following composition:

Rutin:	12.1 area percent,
Isoquercetin:	76.6 area percent,
Quercetin:	10.5 area percent.

The comparison example shows that if water alone is used as solvent less solids (yellow crystals) are obtained and they moreover contain more educt and more byproducts than when a solid mixture that consists of water and an organic solvent is used.

Claims

1. A method for enzymatic cleavage of rutinosides while obtaining rhamnose and/or the corresponding glucopyranosides, which is characterized by the fact that the reaction is carried out in the presence of a solvent mixture of water and one or more organic solvents.

2. A method as in claim 1, which is characterized by the fact that the reaction is carried out at a reaction temperature of 15-80°C.

3. A method as in one of Claim 1 or 2, which is characterized by the fact that the reaction is carried out at a pH value of 3-8.

4. A method as in one of Claims 1-3, which is characterized by the fact that the pH is adjusted with the aid of a buffer system.

5. A method as in claim 4, which is characterized by the fact that the pH value is adjusted with the aid of an aqueous citrate buffer.

6. A method as in one of Claims 1-5, which is characterized by the fact that the reaction is carried out in the presence of one or more organic solvents from among acetates, methanol, ethanol, methyl tert-butyl ether, toluene.

7. A method as in claim 6, which is characterized by the fact that the reaction is carried out in the presence of one or more acetates.

8. A method as in claim 7, which is characterized by the fact that the reaction is carried out in the presence of methyl acetate.

9. The use of campherol glucoside, isoquercetin and/or isorhamnetin glucoside in the food and cosmetics industries.

INTERNATIONAL SEARCH REPORT

Interna al Application No
PCT/EP 99/07686

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12P19/02 C12P19/44 A23L1/00 A61K7/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12P A23L A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DATABASE WPI Section Ch, Week 198706 Derwent Publications Ltd., London, GB; Class B03, AN 1987-040883 XP002128152 & JP 62 000292 A (KANEGAFUCHI CHEM KK), 6 January 1987 (1987-01-06) abstract --- -/--	1-8

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
22 March 2000	30. 03. 00

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Douschan, K

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INTERNATIONAL SEARCH REPORT

Interna. al Application No
PCT/EP 99/07686

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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INTERNATIONAL SEARCH REPORT

Interna .al Application No
PCT/EP 99/07686

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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INTERNATIONAL SEARCH REPORT

Internal Application No
PCT/EP 99/07686

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE WPI Section Ch, Week 199433 Derwent Publications Ltd., London, GB; Class B05, AN 1994-269371 XP002133795 & JP 06 199695 A (KATO K), 19 July 1994 (1994-07-19) abstract -----	9

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP99 /07686

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

See supplemental sheet
No additional fees are to be reimbursed as a result of the findings of the preliminary examination under Rule 40.2(e) PCT.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP99 /07686

The International Searching Authority found that this international application contains multiple inventions, as follows:

1. Claims Nos. 1-8

Claims Nos. 1-8 relate to a method for the production of rhamnose and/or glucopyranosides by enzymatic splitting of rutinosides, whereby the reaction is carried out in the presence of a solvent mixture made up of water and one or several solvents.

2. Claim No. 9

Use of kaempferolglucoside, isoquercetin and/or isorhamnetinglucoside in the food and cosmetics industry.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Internal Application No
PCT/EP 99/07686

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